APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1992, p. 3701–3708 Vol. 58, No. 11
0099-2240/92/113701-08$02.00/0

Methylosinus trichosporium OB3b Mutants Having Constitutive Expression of Soluble Methane Monooxygenase in the Presence of High Levels of Copper

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Received 16 March 1992/Accepted 24 August 1992

The methanotrophic bacterium *Methylosinus trichosporium* OB3b is unusually active in degrading recalcitrant haloalkanes such as trichloroethylene (TCE). The first and rate-limiting step in the degradation of TCE is catalyzed by a soluble methane monooxygenase (sMMO). This enzyme is not expressed when the cells are grown in the presence of copper at concentrations typically found in polluted groundwater. Under these conditions, *M. trichosporium* OB3b expresses a particulate form of the enzyme (pMMO), which has a narrow substrate specificity and does not degrade TCE at any significant rate. We have isolated *M. trichosporium* OB3b mutants that are deficient in pMMO and express sMMO constitutively in the presence of elevated concentrations of copper. One mutant (PP358) exhibited a TCE degradation rate which was almost twice as high as that of the wild-type strain grown under optimal conditions (without copper). All of the mutants lost the ability to express pMMO activity and to form stacked intracellular membranes characteristic of wild-type cells expressing pMMO.

Chlorinated aliphatic solvents represent a major class of pollutants because of their widespread distribution in the environment and their toxic or carcinogenic effects. They are produced in large quantities and are the most frequently detected organic pollutants found in drinking water from groundwater sources (12). Trichloroethylene (TCE), an especially common contaminant, is a known carcinogen in animals and a suspected carcinogen in humans (20). It is resistant to biodegradation in aquifer environments and persists for extended periods of time (27).

Methanotrophic bacteria have been shown to be unusually active in degrading TCE (35). These microbes normally grow with methane as the sole carbon and energy source. Although TCE cannot serve as a sole carbon source, it is cometabolized by methylotrophic bacteria under aerobic conditions, resulting in up to 99% removal of the TCE from sources containing concentrations as high as 50 mg/liter (13).

Well over 100 strains of methanotrophic bacteria have been isolated. Of these, *Methylosinus trichosporium* OB3b has been found to degrade TCE at a rate at least 1 order of magnitude greater than those observed with other pure or mixed cultures (16). The key enzyme responsible for the high rates of TCE degradation is a methane monooxygenase (MMO), the enzyme that catalyzes the first and chemically the most difficult step in methane oxidation. The substrate specificities of bacterial MMOs have been found to be broad, and the specificity of the soluble form of the enzyme (sMMO) generally is broader than the particulate form (pMMO) found associated with stacked internal membranes (3, 8, 28, 33). The sMMO form is not frequently found in methanotrophic bacteria but has been reported in *M. trichosporium*, *Methylosinus sporium* 5, *Methylococcus capsulatus*, and *Methylobacterium* sp. strain CRL26 (3, 29). After incubation with whole cells of *M. trichosporium* OB3b, or purified sMMO with added NADH reductant, TCE is more than 90% mineralized to CO_2 and Cl^- (26). The particulate enzyme activity exhibits negligible rates of TCE degradation (26, 34).

Several studies have demonstrated that the soluble MMO is expressed only in conditions of copper limitation; in copper-sufficient media, the particulate form of the enzyme is preferentially expressed (4, 32). Unfortunately, the concentrations of copper that suppress the expression of sMMO are within the range found in polluted groundwater sources. Although unpolluted sources of groundwater generally contain less than 0.15 μM copper (1, 14), concentrations of copper as high as 7.4 μM have been detected in polluted groundwater (14). Polluted groundwater in the United Kingdom was found to contain 1.5 μM copper, with a standard deviation of 0.5 μM (1). The concentration of copper that effects loss of sMMO expression in *M. trichosporium* OB3b has been reported to be as low as 0.25 μM (3).

Under these conditions, sMMO cannot be produced, thus posing a major limitation in the use of *M. trichosporium* OB3b in bioremediation. To overcome this problem, we have produced mutants capable of expressing sMMO constitutively in the presence of high levels of copper. We have found that these copper-resistant mutants grow well and express sMMO when cultured in the presence of up to 12 μM copper. Under these conditions, one mutant (PP358) exhibited a TCE degradation rate that was almost twice as high as that of the wild-type strain grown in the absence of copper. The mutants did not exhibit any pMMO activity and had lost the ability to form stacked intracellular membranes that is characteristic of wild-type cells expressing pMMO. Differences in the genetic stability and the pattern of proteins present in the soluble and particulate fractions indicate that at least two different classes of mutation are responsible for expression of sMMO in the presence of copper.

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MATERIALS AND METHODS

Cultures. *M. trichosporium* OB3b was obtained from the American Type Culture Collection (ATCC 35070). One of the copper-resistant mutants, PP358, has been deposited in the American Type Culture Collection (ATCC 55314). Wild-type and mutant cultures were grown in Higgins minimal nitrate salts (HMNS) medium (6) supplemented with 2 μM EDTA and cupric sulfate at the indicated levels. Plating media were supplemented with 1.8% Noble agar (Difco), a purified grade of agar. Noble agar was found to support less fungal contaminant growth than less-pure grades of agar, a persistent problem when culturing for weeks in closed containers. Plates were also prepared with 0.1 mg of cycloheximide per ml to prevent fungal contamination. Since *M. trichosporium* OB3b has been reported to be unusually resistant to nalidixic acid (23), cross-contamination of *M. trichosporium* OB3b with other methanotrophs was avoided by supplementing media with 0.05 mg of nalidixic acid per ml.

Stock cultures were maintained by subculturing on agar plates every 3 weeks, and the plates were incubated in desiccators at room temperature with an atmosphere of approximately 50% methane.

Liquid cultures were prepared in sealed bottles or vials in which the liquid phase did not exceed 10% of the total volume. Liquid cultures were prepared by looping a heavy suspension of cells from plates and were subcultured with a 4% (vol/vol) inoculum. Methane and oxygen were supplemented daily in the headspace at 25% (vol/vol) each.

Since methanotrophs are incapable of growth in rich medium, culture contamination by heterotrophic and facultative methanotrophic microbes was detected by colony formation on plate count agar (Difco).

Mutagenesis and isolation of mutants. Liquid cultures grown in HMNS with 0.5% methanol as the sole carbon source were plated on HMNS medium supplemented with 0.025% yeast extract, 5 μM cupric sulfate, and 1% methanol. Yeast extract was found to both improve plating efficiency and promote growth of colonies on methanol, and 5 μM copper was found to prevent expression of sMMO in colonies (data not shown). Plates were incubated in an 11-liter desiccator to which 0.6 ml of dichloromethane (DCM) and 1.0 ml of methanol were delivered every 3 days. Controls were incubated in desiccators supplemented with methanol alone.

After 5 weeks of growth in the presence of DCM, colonies were streaked onto HMNS plates. Expression of MMO in DCM-resistant colonies was assessed by growth with methane as the sole carbon source. For rapid screening of sMMO in the presence of copper, an in situ plate assay was used, and the presence of sMMO was verified by enzyme assays of liquid cultures (see below). Reversion of the DCM-resistant phenotype was evaluated by plating on DCM plus methanol under conditions described above for the mutagenesis procedure.

Culture density. The A_{600} of a cell suspension was used to estimate biomass. A conversion factor of 400 mg (dry weight) of total suspended solids (TSS) per liter per absorbance unit was determined from gravimetric measurements.

Subcellular localization of MMO activity. Cultures were harvested in the late exponential phase by centrifugation at 8,000 × *g* for 20 min at 4°C and washed in cold 25 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.2). The pellets were frozen and stored at −70°C. Cells were thawed and resuspended in lysis buffer containing 5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 7.0) and 5 mM $MgCl_2$ and disintegrated by two passages through a French press at 2,000 lb/in^2. The cellular debris was removed by centrifugation at 5,000 × *g* for 20 min at 4°C. The crude lysates were fractionated into soluble and particulate fractions by centrifugation at 115,000 × *g* for 50 min at 4°C. The membrane pellets were washed and resuspended in cold lysis buffer. MMO activities were determined in the soluble and particulate fractions by propene oxidation as described below.

sMMO activity: TCE degradation by whole cells. The particulate enzyme exhibits negligible rates of TCE oxidation (26, 34), and TCE disappearance was monitored as a direct measurement of sMMO levels in whole cells. Cultures were adjusted to a density of 0.06 to 0.12 absorbance unit in 25 mM MOPS and 2 mM formate (pH 7.2) at 24°C to a final volume of 16 ml in a 40-ml vial. The vial was sealed with a Teflon-coated septum, and 0.01 mg of TCE was added from an aqueous stock solution. The concentration of TCE in the liquid phase was estimated to be 1 mg/liter from the gaseous concentration by using a value of the Henry's law constant of 0.4 at 24°C. TCE levels were measured by gas chromatography analysis of the headspace on a fused silica capillary column (30 m by 0.53 mm) with a DB-624 stationary phase having a film thickness of 0.003 mm (J&W Scientific). The column was operated at 70°C with a flame ionization detector.

sMMO activity: naphthalene oxidation by whole cells. A rapid and simple colorimetric method was developed to detect sMMO activity by virtue of its ability to oxidize naphthalene to naphthol. The pMMO enzyme is not active in naphthalene oxidation. This method differs from a previously published method (3) in two ways: naphthalene is presented to cells in a detergent-solubilized form, and the naphthol dye-colored product is stabilized by acidification. Briefly, 1% (wt/vol) naphthalene was dissolved in 20% (vol/vol) Pluronic L-62 surfactant (BASF Corp.) The reaction was initiated by adding the naphthalene stock to a cell suspension at 24°C having a density of 40 to 100 mg of TSS per liter at a ratio of 1:100. The reaction was terminated after 30 min of incubation at room temperature by centrifugation at 8,800 × *g* for 5 min. The naphthol concentration in the supernatant was determined by a standard colorimetric procedure (31) by using Fast Blue BN dye (tetrazotized *o*-dianisidine; Sigma). In this case, 0.1 ml of a fresh 1% solution of Fast Blue BN was added. The color formation was stabilized after 15 s by adding 0.4 ml of glacial acetic acid, and naphthol was quantitated by comparison with the A_{540} of naphthol standards.

In situ sMMO activity: naphthalene oxidation by colonies. Expression of sMMO in colonies growing on agar plates was detected by a naphthalene agar overlay assay as follows. A 1% naphthalene solution in 20% Pluronic L-62 surfactant was diluted 1:50 in a 1% solution of low-melting-point agarose (electrophoresis grade; Bethesda Research Laboratories) containing 25 mM MOPS (pH 7.2). After the solution was cooled to 35°C, colonies growing on agar plates were covered by 5 ml of the naphthalene agarose solution. Once the agarose had solidified, plates were incubated at room temperature for 1 hour in a chamber containing 50% oxygen. Naphthol formation was detected by covering the plates with 1.0 ml of a fresh solution of 1% Fast Blue BN. Colonies expressing sMMO developed a dark red color. Color formation was stabilized by adding 1.0 ml of glacial acetic acid after 5 min at room temperature.

In vitro MMO activity: propene oxidation. MMO activity

was determined in the soluble and insoluble cell fractions by the oxidation of propene to propene oxide (4). Cell fractions prepared by centrifugation as described above were diluted in 25 mM PIPES (pH 7.0) buffer to a protein concentration of 5 to 7 mg/ml (soluble fraction) or 2 to 3 mg/ml (particulate fraction). Assays were performed in 9-ml vials containing 0.8 ml of sample and a 1:1 mixture of air and propene in the headspace at 24°C. The reaction was initiated by adding NADH to a final concentration of 5 mM. Propene oxide was assayed by gas chromatography analysis of the liquid phase by using the same column used for the TCE analysis but operating it at 47°C.

Protein determination. Protein was assayed by the Bradford method (2) by using bovine serum albumin as a reference standard.

Protein gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (22). Lanes were loaded with approximately 10 μg of protein and stained with Coomassie blue.

Electron microscopy. Cultures were harvested in mid-exponential phase and fixed at 4°C for 1 h in 0.8% glutaraldehyde and 0.7% osmium tetroxide in 0.1 M cacodylate buffer (10). After staining in 2.5% uranyl acetate in water for 1 h, cells were washed and dehydrated through a graded ethanol series. Embedding was done in Spurr's resin. After staining with aqueous 2.5% uranyl acetate and Reynolds lead citrate, thin sections were examined on a JEOL 100CX transmission electron microscope operated at 80 kV.

RESULTS

Mutagenesis. DCM was found to act as a suicide metabolite to cells expressing either sMMO or pMMO, presumably because it becomes oxidized by either enzyme to carbon monoxide, a toxic intermediate (7). In addition, DCM has been shown to have a direct mutagenic effect in *Salmonella* spp. by the Ames test without the need for biochemical activation (17, 21). Thus, DCM is able to act as both a mutagen and a selection agent against methanotrophs expressing MMO. In earlier studies, mutants selected for resistance to DCM were found to lack both the soluble and the particulate enzymes and thus could not grow with methane as the carbon source (24, 25).

To select for mutants deficient only in the particulate form of MMO, we modified the DCM selection procedure in a number of ways. To suppress sMMO expression, the plating medium of the selection plates was supplemented with a high level of copper. Also, since both methanol and DCM are oxidized by sMMO, excess methanol was included with each DCM addition to inhibit possible sMMO-mediated activation of DCM to toxic oxidation intermediates in any colonies where sMMO was being expressed. Finally, yeast extract was added to improve plating efficiency and growth on methanol.

DCM survivors appeared slowly, and the mutagenesis selection was carried out for a long period of time (5 weeks). Survival on DCM plus methanol was low: approximately 5 in 10^5 CFU survived, as compared to the methanol controls. This mutation rate is low, but rates of mutagenesis in methanotrophs have been reported to be low in several reports (for a review, see reference 11).

In 65 surviving colonies, approximately one-third failed to grow on plates incubated with methane. This phenotype was the same as that reported previously (25) in which DCM selection of *M. trichosporium* OB3b on plates grown in methanol resulted in mutants deficient in both pMMO and sMMO. However, the majority of the DCM-resistant mutants (44 colonies) from our mutagenesis retained the ability to grow on methane as the sole carbon source.

Mutants retaining MMO activity may either be revertants to the wild-type phenotype or they may be defective in one but not both forms of the enzyme. Thus, the mutants were further classified by evaluating the expression of sMMO in the presence of copper. An in situ plate assay based on the oxidation of naphthalene by sMMO showed that nearly half of the surviving colonies were expressing active sMMO in HMNS medium even in the presence of 5 μM copper. The remainder of the DCM-resistant MMO^+ mutants appeared to have the wild-type phenotype in that expression of sMMO was not detected in cultures grown in copper-containing medium.

To determine the effects of copper on the levels of expression of sMMO, all 44 DCM-resistant MMO^+ mutants were grown in liquid culture. Many mutants had great difficulty in adapting to growth in liquid culture, having lag times as long as 3 weeks. Generally, the mutants with the longest lag times were later found to have the best sMMO expression in the presence of copper. A rapid screen of those mutants that grew in liquid culture in the presence of 2 μM copper showed that 19 expressed sMMO activity. Wild-type cultures had levels of sMMO activity averaging 3.8 μg of naphthol h^{-1} optical density unit $(ODU)^{-1}$ when grown in the absence of copper, and sMMO activity was not detectable when grown in the presence of copper. Cultures of the 19 mutants had sMMO activities averaging 9.6 μg of naphthol h^{-1} ODU^{-1} when grown in the absence of copper and 4.3 μg of naphthol h^{-1} ODU^{-1} when grown in the presence of 2 μM copper. One mutant, strain PP358, had unusually high sMMO activities: 13.6 μg of naphthol h^{-1} ODU^{-1} when grown without copper and 16.4 μg of naphthol h^{-1} ODU^{-1} in copper-grown cultures.

Stability of the copper-resistant sMMO mutants. Of the 19 mutant strains that expressed sMMO in the presence of copper, several grew very slowly in the presence of copper or appeared to be genetically unstable, losing the copper-resistant sMMO phenotype after subculturing in copper-containing medium. Five of the 19 mutant strains both grew well and continued to express sMMO in copper-containing medium upon continuous subculturing. Three strains, PP323, PP333, and PP358, have retained the copper-resistant phenotype for more than 11 months of subculturing. The other two reverted to pMMO expression after 5 (PP319) and 7 (PP311) months.

Attempts to grow the five most-stable copper-resistant sMMO mutants on DCM-plus-methanol plates under the original selection conditions have been unsuccessful. This strongly suggests that growth with methane as the sole carbon source on plates and in copper-containing liquid medium has further selected a new phenotype following the original DCM-resistant phenotype.

Level of copper tolerance. To evaluate the limits of copper concentrations in which mutants continue to express sMMO, cultures were grown in liquid medium containing copper at concentrations ranging from 2 to 12 μM. Mutants PP311 and PP319 reproducibly exhibited a lag of 5 to 6 days before the onset of growth in media containing 12 μM copper (Fig. 1). Although mutants PP323, PP333, and PP358 initially showed difficulty growing in the presence of 12 μM copper, when subcultured in the presence of 5 μM copper, they became more tolerant towards growth in higher concentrations of copper and then were able to grow without a noticeable lag period in media containing 12 μM copper.

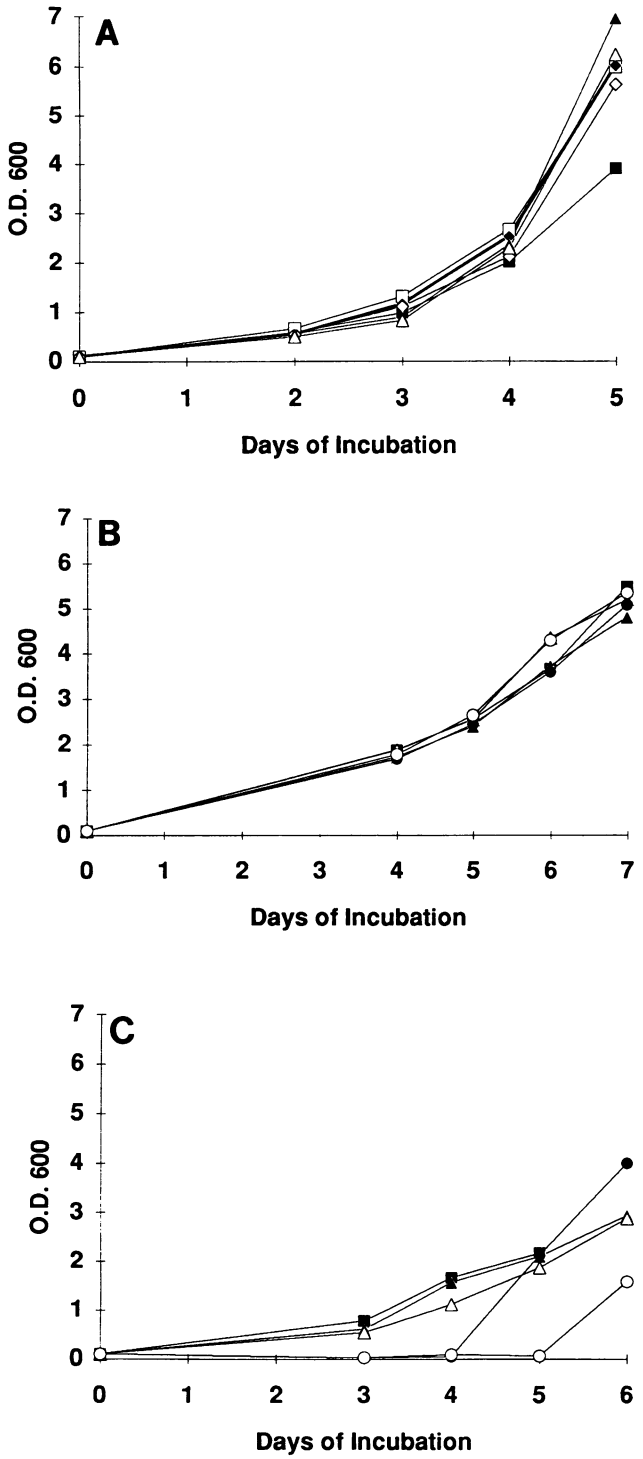

FIG. 1. Effects of various concentrations of copper in culture medium on growth of wild-type and copper-resistant mutants. Culture density was determined by the OD at 600 nm. (A) Wild-type OB3b; (B) mutant PP358; (C) mutant PP311. Symbols: ■, no copper; □, 0.2 μM copper; ◆, 0.5 μM copper; ◇, 1.0 μM copper; ▲, 2.0 μM copper; △, 5.0 μM copper; ●, 10.0 μM copper; ○, 12.0 μM copper.

TABLE 1. Levels of sMMO expression in whole cells of the wild type or mutants grown in medium containing 5 μM copper

Culture	sMMO activity (liters of TCE day^{-1} mg of TSS^{-1})	
	Grown without Cu	Grown in 5 μM Cu
Wild type	0.8 (±0.3)[a]	0.03 (±0.06)[a]
Mutants		
PP311	1.0 (±0.1)	1.1 (±0.3)
PP319	1.1 (±0.2)	1.1 (±0.4)
PP323	1.0 (±0.4)	1.3 (±0.5)
PP333	1.0 (±0.3)	1.3 (±0.4)
PP358	1.5 (±0.1)	1.4 (±0.3)

[a] Values in parentheses indicate standard deviations from averages of at least three different cultures.

This apparent ability to adapt towards growth at the higher concentrations of copper was not found in mutants PP311 and PP319.

The five stable mutants expressing sMMO constitutively with respect to copper concentrations had approximately the same specific activity of sMMO, as assayed by TCE decay, regardless of whether they were grown in the absence or presence of 5 μM copper (Table 1). The average activity of the wild-type cultures was 0.8 liter of TCE day^{-1} mg of TSS^{-1}, with a standard deviation of 0.3 liter of TCE day^{-1} mg of TSS^{-1}. This value is similar to data reported earlier (34) in which wild-type cultures were assayed under similar conditions and yielded rate constants of 0.52 to 0.54 liter of TCE day^{-1} mg (dry weight)$^{-1}$. Generally, all of the cultures of mutant strains had higher sMMO activities than did wild-type cultures. Mutant cultures did not have reduced levels of sMMO activity when grown in the presence of 5 μM copper. Two of the mutants, PP311 and PP319, had lower sMMO specific activities when grown at concentrations of copper exceeding 10 μM (Fig. 2). The other three mutants, PP323, PP333, and PP358, maintained the same specific activity regardless of copper concentrations in the growth medium. By way of comparison, the wild-type cultures failed to express sMMO at much lower concentrations of copper. Generally, sMMO activity was not detected in wild-type cultures grown at concentrations of copper above

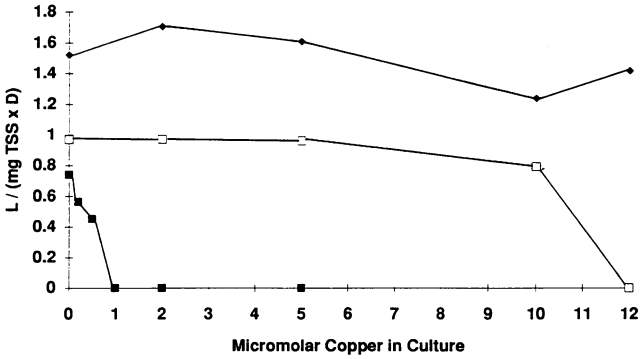

FIG. 2. Effects of various concentrations of copper in culture medium on expression of sMMO in wild-type and copper-resistant mutants. Enzyme activity of whole cells was assayed by TCE degradation at 1 mg of TCE/liter and reported as a pseudo-first-order rate constant (liters of TCE day^{-1} milligrams of TSS^{-1}). Symbols: ■, wild type; □, mutant PP358; ◆, mutant PP311.

TABLE 2. Subcellular localization of MMO activity measured by propene oxidation of soluble and particulate fractions

Culture	% of total MMO activity in particulate fraction	
	Cultured without Cu	Cultured in 5 μM Cu
Wild type	8	90
Mutants		
PP311	<0.1	0.15
PP319	0.11	2.1
PP323	0	0
PP333	2.0	0
PP358	0	0
PP319R	0.70	100

0.5 μM in batch culture, depending on the density of the culture (Fig. 2).

Subcellular localization of MMO. Wild-type OB3b cultured in 5 μM copper expressed MMO almost entirely in the particulate fraction, while copper-deficient cultures expressed MMO almost entirely in the soluble fraction (Table 2). In contrast, mutants grown in 5 μM copper expressed MMO almost entirely in the soluble fraction. A revertant of PP319 (PP319R) was found to have lost expression of sMMO in copper-containing medium and had acquired the wild-type distribution of particulate MMO when cultured in the presence of 5 μM copper.

Electron microscopy. Wild-type OB3b cultures grown in media containing 5 μM copper were found to have type II intracellular membranes lying peripherally in lamellar stacks roughly parallel to the cell wall (Fig. 3). When grown in the absence of copper, wild-type OB3b lacked stacked membranes but frequently contained distinct membrane-bound vesicles that were not organized in tightly stacked membranes. These membrane-bound vesicles were observed earlier in electron micrographs of *M. trichosporium* OB3b (8, 30). The five stable mutants grown in 5 μM copper did not have stacked internal membranes, but the membrane-bound vesicles found in wild-type cultures were present. The revertant PP319R, on the other hand, had regained the ability to form internal stacked membranes when grown in the presence of copper (Fig. 3).

SDS-PAGE. The protein compositions of the soluble and particulate fractions of the mutants were significantly different from those of the wild type. The three polypeptides of the hydroxylase component of sMMO (15), having molecular weights of 54,000, 45,000, and 23,000, appeared as prominent bands in SDS-PAGE of the soluble fraction of wild-type cells cultured in the absence of copper (Fig. 4). These bands were also found in the soluble fractions of all of the mutants cultured without copper. When cultured in the presence of 5

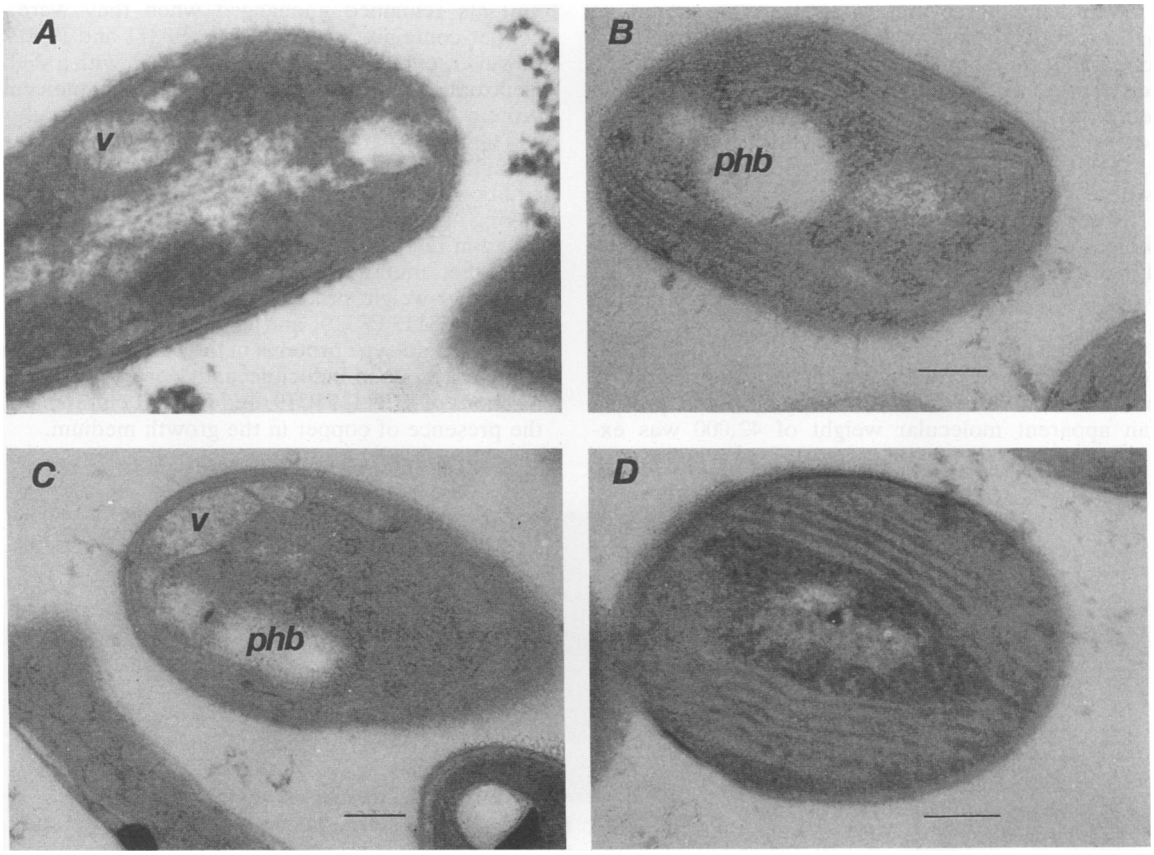

FIG. 3. Electron micrographs of cultures of wild-type and mutant cells of *M. trichosporium* OB3b. (A) Wild type grown without copper addition; (B) wild type cultured in 5 μM copper; (C) mutant PP358 cultured in 5 μM copper; (D) revertant PP319R cultured in 5 μM copper-containing medium. Wild type grown in copper-deficient medium and PP358 grown in copper-containing medium contain vesicles (v) but lack peripheral stacked membranes found in wild type and PP319R revertant grown in copper-containing medium. Granules of polyhydroxybutyrate (phb) are apparent.

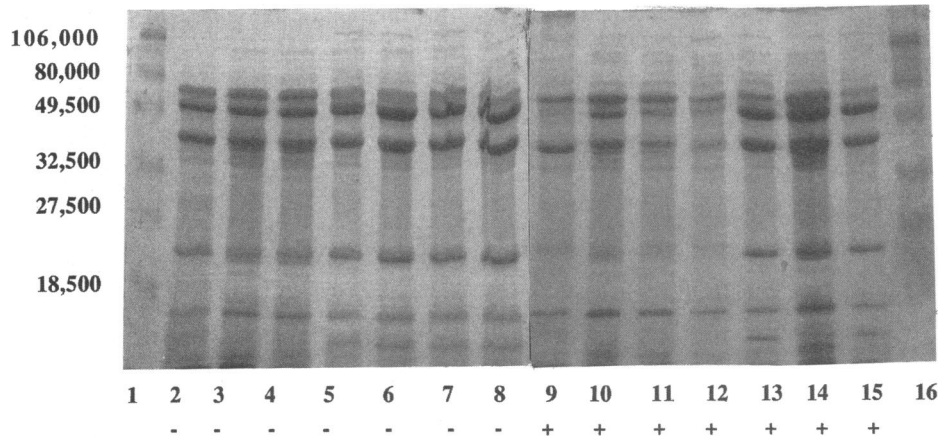

FIG. 4. SDS-PAGE of the soluble fractions of wild-type and mutant cells: effects of copper supplementation of growth medium. Samples are from cultures grown without copper (−) or with 5 μM copper supplementation (+). Molecular weights of standards (lanes 1 and 16) are 106,000, 80,000, 50,000, 32,500, 27,500, and 18,500. Lanes: 2 and 9, wild-type cultures; 3 and 10, PP311; 4 and 11, PP319; 5 and 12, PP319R; 6 and 13, PP323; 7 and 14, PP333; 8 and 15, PP358.

μM copper, however, wild-type OB3b cells no longer have the prominent hydroxylase bands, but all three hydroxylase polypeptides continue to be prominent bands in the soluble fractions of mutants PP323, PP333, and PP358 grown in copper. These same three mutant strains also exhibit an additional prominent band with an apparent molecular weight of 15,500, the approximate molecular weight of the sMMO component B. High expression of this protein was not observed in the soluble fractions of wild-type, PP311, PP319, or revertant PP319R cultures. Although the other mutants, PP311, PP319, and PP319R, also expressed large amounts of all three of the hydroxylase components in the absence of copper, they were deficient in the prominent 23,000-molecular-weight hydroxylase gamma band when cultured in 5 μM copper (Fig. 4).

In the particulate fraction, wild-type OB3b cultured in the absence of copper expressed high levels of proteins having apparent molecular weights of 80,000 and 82,000 (Fig. 5). The intensity of these protein bands was decreased in wild-type OB3b cultured in 5 μM copper, but a protein having an apparent molecular weight of 42,000 was expressed in large amounts. The proteins found in the particulate fractions of mutants PP323, PP333, and PP358 were similar to those of the wild-type proteins from copper-deficient cultures, except that the expression pattern in the mutants remained unchanged when they were grown in copper-containing medium. The PP311 and PP319 mutants, however, expressed a dominant protein with a slightly higher electrophoretic mobility than the 42,000-molecular-weight protein found in copper-grown wild-type cultures. This 40,000-molecular-weight protein was reproducibly observed in different cultures of PP311, PP319, and PP319R. In addition, PP311 and PP319 expressed high levels of a protein having an apparent molecular weight of 24,000 that was not found in the particulate fractions of the wild-type or any of the other mutant cultures. Both the 40,000- and 24,000-molecular-weight proteins found in the particulate fractions of the PP311, PP319, and PP319R mutant cultures differed from the wild-type proteins in the pattern of expression: they were not copper inducible and were found in particulate fractions of PP311, PP319, and PP319R cultures regardless of the presence of copper in the growth medium.

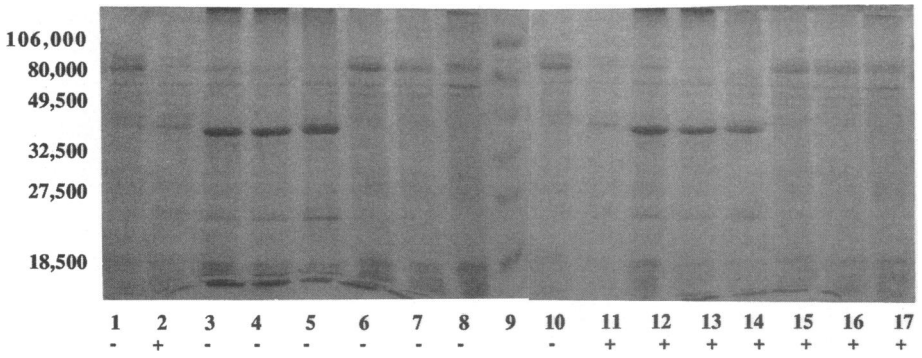

FIG. 5. SDS-PAGE of the particulate fractions of wild-type and mutant cells: effects of copper supplementation of growth medium. Samples came from cultures lacking copper (−) or from cultures grown in 5 μM copper (+). Molecular weights of standards (lane 9) are 106,000, 80,000, 50,000, 32,500, 27,500, and 18,500. Lanes: 1, 2, 10, and 11, wild-type cultures; 3 and 12, PP311; 4 and 13, PP319; 5 and 14, PP319R; 6 and 15, PP323; 7 and 16, PP333; 8 and 17, PP358.

Finally, there were no obvious differences in the patterns of protein expression of strains PP319 and PP319R, even though the revertant had acquired the wild-type phenotype of copper-induced expression of pMMO and the ability to form internal stacked membrane arrays.

DISCUSSION

The discovery of high rates of TCE degradation by methanotrophs has made their practical application promising for bioremediation of highly recalcitrant chlorinated solvents in polluted groundwater and leachates. The activity of the sMMO of *M. trichosporium* OB3b has been found to be at least 1 order of magnitude greater than that of any other pure or mixed culture (18). However, practical applications using this methanotroph in the remediation of groundwater contaminated with chlorinated solvents are hampered by the repression of sMMO activity by micromolar concentrations of copper.

By modifying a technique used previously to obtain mutants of methanotrophs deficient in all forms of MMO (24, 25), we successfully obtained mutants selectively deficient in only the particulate form of MMO. Through our modified selection strategy, a large proportion of mutants were found to have selectively lost the ability to express pMMO; in addition, about 29% (19 of 65 DCM-resistant mutants) continued to express sMMO when grown in the presence of copper.

The majority of the copper-resistant sMMO mutants grew poorly and were genetically unstable; 14 of 19 failed to grow well and reverted to the wild-type phenotype when subcultured in copper-containing liquid medium. A stable copper-resistant phenotype of the $sMMO^+$ $pMMO^-$ mutants arose only after long periods of adaptation, after which the mutants had lost their original DCM-resistant phenotype. This result strongly suggests that growth on plates and in copper-containing liquid medium during the mutant isolation and characterization steps has further selected a new phenotype in five mutant strains, different from the original DCM-resistant phenotype.

The five stable mutants appear to belong in two different classes with respect to a number of criteria. First, they differed in growth rates and expression levels of sMMO in medium containing 12 μM copper. Under these conditions, strains PP311 and PP319 grew more slowly and expressed reduced levels of sMMO activity, while strains PP323, PP333, and PP358 were able to adapt towards wild-type growth rates in 12 μM copper and expressed high levels of sMMO at all levels of copper. Secondly, this latter group of mutants appears to be exceptionally stable and has not reverted after 11 months of continuous subculturing. Reversion to pMMO expression was detected in the members of the other group, namely, in PP319 after 5 months and in PP311 after 7 months of serial subculturing.

Both groups of these mutants have completely lost their ability to express pMMO, since MMO activity was not found in the particulate fractions of mutant cultures grown in 5 μM copper. At this concentration of copper, wild-type cultures expressed most of the MMO activity in the particulate fraction. A revertant in copper-resistant expression of sMMO, PP319R, was found to have acquired pMMO expression; all of its MMO activity was found in the particulate fraction when cultured in media containing 5 μM copper.

Lastly, the two classes of sMMO-constitutive mutants differed in patterns of protein expression. In PP311 and PP319, there appeared to be reduced levels of sMMO subunit expression in cultures grown in the presence of copper, especially in the gamma subunit of the sMMO hydroxylase subunit. In addition, particulate fractions from these two mutants have an unusual pattern of protein components not found in the wild type. On the other hand, mutants PP323, PP333, and PP358 appear to have lost any copper inducibility in the pattern of protein expression in both the soluble and particulate fractions. The patterns of proteins found in the particulate and soluble fractions of these mutants appear to be the same as those found in the wild type cultured in the absence of copper.

The presence of internal stacked membranes in methylotrophic bacteria has been tightly correlated with the presence of MMO activity (19). In methanotrophs that possess both sMMO and pMMO, expression of pMMO is correlated with the presence of internal stacked membranes (9, 30). In this study, mutant cells lacking pMMO activity were found to be also lacking in intracellular stacked membranes, further confirming this dependence of pMMO activity on this internal membrane structure. In addition, revertant PP319R regained both expression of pMMO activity and the ability to form stacked internal membranes.

The loss of pMMO activity may be a result of a defect in a structural gene of the enzyme or a result of the disruption of a gene whose product is necessary for membrane morphogenesis. It is also possible that the loss of pMMO expression is due to the loss of a regulatory gene or a defect in copper uptake in these mutants, eliminating copper-induced gene expression. Preliminary results, however, suggest that copper uptake and copper-induced regulation of protein expression are not the key defects of any of these copper-resistant mutants (28a).

Studies are currently under way to characterize the actual nature of the defects in the different classes of copper-resistant mutants. The recent cloning of the sMMO locus should facilitate the genetic analysis of the mutants (5). In parallel, antibiotic-resistant variants of copper-resistant mutants are being used in lab-scale bioreactors to investigate their ability to maintain culture purity and high levels of remediation of TCE-contaminated water under simulated field conditions (15a).

ACKNOWLEDGMENTS

This work was supported in part by the Texas Advanced Technology Program under grant number 003658-050.

The electron micrographs were prepared by Angel Paredes of the Cell Institute, Austin, Tex.

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